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Value of matrix-assisted laser desorption ionization-time of flight for routine identification of viridans group streptococci causing bloodstream infections

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Abstract

Phenotypic tests do not always unequivocally identify some species of viridans group streptococci (VGS). *sodA* sequence analysis is the most accurate method for identification, although it requires specialized personnel and has not been applied systematically in clinical microbiology laboratory routines. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) is emerging as a rapid alternative for bacterial identification. This study assesses the ability of MALDI-TOF and the API 20 Strep system to identify VGS isolates recovered from blood cultures using *sodA* sequence analysis as the reference method. All clinically significant VGS isolates recovered from blood cultures between January 2007 and January 2010 were identified by *sodA* sequence analysis and API 20 Strep. The strains were then tested by MALDI-TOF. Agreement between API 20 Strep/MALDI-TOF and *sodA* sequence analysis was determined. We examined 124 clinical isolates. Sensitivities of API 20 strep and MALDI-TOF for the species level identification of VGS isolates were, respectively, as follows: 60.5% and 73.4%. Sensitivities of API 20 strep and MALDI-TOF for the group level identification were, respectively, as follows: 70% and 94.3%. The turnaround times to identify VGS isolates by *sodA* sequence analysis, API 20 Strep and MALDI-TOF were 12–24, 24–48 h and 15 min, respectively. API 20 Strep cannot accurately identify all isolates of VGS. MALDI-TOF appeared to be a rapid and reliable alternative for identification of VGS strains to group level, but was not able to discriminate closely related species of certain groups.

Keywords: API 20STREP, biochemical identification, MALDI-TOF, *sodA* gene, *Streptococcus*, viridans
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Introduction

Identification of viridans group streptococci (VGS) causing bloodstream infections is important for clinical and epidemiological reasons [1]. As VGS species are genetically related, it is difficult to identify them using routine biochemical methods. Therefore, molecular characterization of VGS is mandatory for identification to species level. Phenotypic methods such as the API 20 Strep system remain the most widely used in clinical laboratories; however, 20–30% of strains cannot be unequivocally identified to species level, and the technique does not provide results until 24–48 h after isolation [2,3]. Several nucleic acid–based technologies have been developed to improve identification of VGS. Sequence analysis of the *16S rRNA* gene has been widely applied for the taxonomy of streptococci, although the ability of this method to differentiate between closely related species, particularly *Streptococcus oralis* and *Streptococcus mitis*, has been questioned [4]. The superoxide dismutase gene (*sodA*) has a more discriminative target sequence than the *16S rRNA* gene for differentiating between closely related streptococcal species and is considered the most accurate method for identification of VGS to species level [3–6]. However, *sodA* sequence analysis is very time-

consuming, requires specialized personnel, and has not been applied systematically in clinical microbiology laboratory routines.

Another recently published approach, multilocus sequence analysis (MLSA), has proven to be a reliable alternative for the identification of VGS [7]. However, this technique is not available in most clinical microbiology laboratories and has not been proven to be superior to the *sodA* sequence analysis for the speciation of VGS.

In recent years, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) has emerged as a rapid alternative for bacterial identification based on the protein composition of microbial cells. The few studies that have analysed identification of VGS with this system are limited to the differentiation of species of certain groups, and most use sequence analysis of the *16S rRNA* gene as a comparator. In addition, these studies also report that *S. mitis* group isolates have been misidentified as *Streptococcus pneumoniae* [8–10]. The aim of the present study was to assess the ability of MALDI-TOF and the API 20 Strep system to identify VGS isolates recovered from blood cultures using *sodA* sequence analysis as the reference method.

Material and Methods

Bacterial strains and cultures

All clinically significant VGS isolates recovered from blood cultures between January 2007 and January 2010 in our institution were studied. The isolates were subcultured on chocolate agar and incubated at 37°C in 5% CO_2 for 48 h. The strains were preliminarily identified as VGS based on a negative catalase test result, Gram stain (Gram-positive cocci in chains) and alpha-haemolysis. Optochin and bile solubility tests were used to rule out pneumococci [11,12]. Bile esculin and sodium chloride were used to rule out enterococci. The VGS isolates were identified by *sodA* sequence analysis and by the API 20 Strep system and stored at −70°C. The strains were then thawed and subcultured for analysis by MALDI-TOF.

Reference strains

The reference strains used for quality control were *Streptococcus equinus* CECT 213 (ATCC 33317), *Streptococcus mutans* CECT 479 (ATCC 25175), *Streptococcus pneumoniae* CECT 993 (ATCC 27336), *Streptococcus intermedius* CECT 803 (ATCC 27335), *Streptococcus gordonii* CECT 804 (ATCC 33399), *Streptococcus salivarius* CECT 805 (ATCC 7073), *Streptococcus anginosus* CECT 948 (ATCC 12395) and *Streptococcus oralis* CECT 907 (ATCC 35037).

Phenotypic identification

Phenotyping was performed using the API 20 Strep system (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions. API 20 strep results were obtained after 24–48 h of incubation at 35–37°C as a numerical profile obtained from a visual reading, according to the manufacturer's recommendations. Strains were identified using apiweb (https://apiweb.biomerieux.com/) and API® 20 StrepV3.0 (bioMérieux) and classified as follows: (i) isolates identified to species level, (ii) isolates identified to genus level and (iii) unidentified isolates. Identification to species level was classed as excellent, very good, good and acceptable.

Genotypic identification

DNA extraction. Bacterial DNA was extracted and purified using the QIAmp DNA Minikit (Qiagen Ltd, Crawley, UK) according to the manufacturer's instructions.

sod gene polymerase chain reaction (PCR)-based sequencing. . An internal fragment of approximately 480 bp of the sodA gene was amplified by PCR using degenerated primers d1 (5′-CCITAYICITAYGAYGCIYTIGARCC-3′) and d2 (5′-ARRTARTAIGCRTGYTCCCAIACRTC-3′), as previously described [6]. The amplicons obtained were detected by electrophoresis through a 1.4% agarose gel in 1× Tris-borate-EDTA buffer and visualized using ethidium bromide staining and UV transillumination. After purification in a commercial system (High Pure PCR Product Purification Kit; Roche Diagnostics, Mannheim, Germany), PCR products were subsequently sequenced using the Big Dye Terminator method according to the manufacturer's instructions and detected in an AbiPrism 3100 automatic DNA sequencer (Applied Biosystems Inc, Foster City, CA, USA). The sequences obtained were compared with those stored in GenBank using BLAST alignment software (http://www.ncbi.nlm.nih.gov/blast) and BIBI (bioinformatics bacterial identification tool: http://pbil.univ-lyon1.fr/bibi). Identification to species level was defined as ≥99% sequence similarity with only one species of Streptococcus. When the score was ≥95% and <99%, the isolate was assigned to the corresponding genus (*Streptococcus* spp.)

Matrix-assisted laser desorption ionization-time of flight mass spectrometry

A small amount of a freshly grown 48-hour-old colony was inoculated directly onto a ground steel MALDI target plate in a thin film. The film was then overlaid with 1 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid and allowed to dry at

room temperature. The plate was inserted into the Microflex MALDI-TOF MS instrument (Bruker Daltonics, Bremen, Germany). The mass spectra generated were analysed using the Bruker Biotyper 3.0 software package and compared with a reference spectra database.

The identification criteria used in our analysis, as outlined by the manufacturer, were as follows: a score of ≥2 indicated identification to species level, a score between 1.7 and 1.9 indicated identification to genus level, and a score ≤1.7 was interpreted as unreliable identification.

Definitions

Discrepant results were classified according to the following definitions.

Very major discrepancies: the isolate was assigned to a different genus.
Major discrepancies: the isolate was assigned to the same genus, to a different graph, and to a different species.
Minor discrepancies: the isolate was assigned to a different species but to the same group.

Results for species level identification by MALDI-TOF and API 20 Strep were considered concordant when identification to species level exactly matched that of the reference method.

Results for group-level identification by MALDI-TOF and API 20 Strep were considered concordant when identification to group level exactly matched that of the reference method.

Statistical analysis

Agreements and validity values were calculated with a 95% confidence interval based on an exact binomial distribution. Agreements between the two methods evaluated and the reference technique were compared using a two-tailed McNemar test for paired samples. A p-value of <0.05 was considered statistically significant. Data were analysed using SPSS, version 15.0 (SPSS Inc, Chicago, IL, USA).

Results

We examined 124 clinical isolates from 115 patients (one strain per episode of bloodstream infection) and eight reference strains.

Reference strains

The identification results of the reference strains obtained using the three methods are shown in Table 1.

Clinical isolates

sodA sequence analysis identified all clinical isolates to species level (≥99% sequence similarity with only one species of *Streptococcus)*. Identification of 124 VGS in blood culture isolates by *sodA* sequence analysis is shown in Table 2.

The API 20 Strep identified 93/124 isolates (75%) to species level. Seventy-six isolates were concordant to species level by *sodA* sequence analysis and API 20 Strep (Table 3). In 18 of the isolates, species identification differed between *sodA* sequence analysis and API 20 Strep (Table 3). Eight of these 18 isolates were matched to group level (minor discrepancies). Agreement between API 20 Strep and *sodA* sequence analysis was 60.5% to species level and 70% to group level (Table 4).

Identification to genus level by the API system was achieved for 19/124 (15.3%) cases; 12/124 (9.6%) isolates remain unidentified (Table 3).

Ninety-one isolates were concordant to species level by *sodA* sequence analysis and MALDI-TOF, 74 with a score ≥2 and 17 with scores between 1.7 and 1.9. Discrepant results were obtained in 27 isolates, 16 with a score of ≥2 and 11 with scores between 1.7 and 1.9 (Table 3). Among these discrepant results, 25 were identical at group level (minor discrepancies). Agreement in identification to species level and to group level between MALDI-TOF and *sodA* sequence analysis was 73.4% and 93.5%, respectively (Table 4). Identification was unreliable in 6/124 (4.8%) isolates (score, ≤1.7).

TABLE 1. Identification results for the reference strains according to the two techniques evaluated

Reference strains	CECT/ATCC	*sodA* sequence analysis (sequence similarity, %)	API 20 strep (ID, %)	MALDI-TOF (score)
S. equinus	213/33317	*S. equinus* (99)	*S.* bovis II4 (99.9)	*S. gallolyticus subsp pasteurianus* (1.8)
S. mutans	479/25175	*S. mutans* (99)	*S. mutans* (99.9)	*S. mutans* (2.3)
S. pneumoniae	993/27336	*S. pneumoniae* (99)	*S. pneumoniae* (89)	*S. pneumoniae* (2.1)
S. intermedius	803/27335	*S. intermedius* (99)	*S. intermedius* (92.3)	*S. intermedius* (1.8)
S. gordonii	804/33399	*S. gordonii* (99)	Unreliable identification	*S. gordonii* (2.1)
S. salivarius	805/7073	*S. salivarius* (99)	*S. salivarius* (98.2)	*S. salivarius* (2.1)
S. anginosus	948/12395	*S. anginosus* (100)	*S. anginosus* (99)	*S. anginosus* (2.1)
S. oralis	907/35037	*S. oralis* (99)	*S. oralis* (78)	*S. pneumonie* (1.8)

ID, identification.

TABLE 2. Identification of 124 VGS strains isolated from blood cultures by *sodA* sequence analysis

Species (*sodA* sequence analysis)	No.
S. mitis	7
S. oralis	25
S. cristatus	3
S. infantis	3
S. salivarius	8
S. vestibularis	1
S. infantarius	3
S. mutans	1
S. anginosus	24
S. constellatus	6
S. sanguis	5
S. parasanguis	10
S. gordonii	4
S. equinus	9
S. gallolyticus subsp *gallolyticus*	5
S. gallolyticus supsp *pasteurianus*	6
S. lutetiensis	4

Incorrect identification by the two techniques evaluated includes minor and major discrepancies (Table 3). No very major discrepancies were observed.

Sensitivity, specificity, negative predictive value and positive predictive value of the two evaluated methods for the identification of VGS isolates are represented in Table 5.

The turnaround times to identify VGS isolates from blood cultures by *sodA* sequence analysis, API 20 strep and MALDI-TOF, were 12–14 h, 24–48 h and 15 min, respectively.

Discussion

Currently, no recognized reference standard technique can satisfactorily identify many VGS. However, in a large number

TABLE 3. Results of API 20 strep and MALDI-TOF for the identification of VGS isolated from blood cultures

		API 20 Strep					MALDI-TOF				
					Discrepant[c]			Concordant[b]		Discrepant[c]	
		Unreliable ID	Genus level ID	Concordant[b]	Major	Minor	Unreliable ID (score > 1.7)	1.7–1.9	≥2	Major	Minor
S. mitis group	38										
S. mitis	7	1	1	5	0	0		0	0	1	6
S. oralis	25	2	4	16	3	0	2	5	10	0	8
S. cristatus	3			3	0	0		2	1	0	0
S. infantis	3		1	1	1	0		2	1	0	0
salivarius group	12										
S. salivarius	8			8	0	0	1	1	5	1	0
S. vestibularis	1	1		0	0	0		0	1	0	0
S. infantarius	3		2	1	0	0		1	2	0	0
mutans group	1										
S. mutans	1			1	0	0		1	0	0	0
anginosus group	30										
S. anginosus	24	3	7	6	2	6	3	2	17	0	2
S. constellatus	6			5	1	0		0	6	0	0
sanguinis group	19										
S. sanguis	5			4	1	0		0	5	0	0
S. parasanguis	10		2	7	1	0		1	9	0	0
S. gordonii	4	2	1	0	0	1		0	4	0	0
bovis group	24										
S. equinus	9	2		6	0	1		0	0	0	9
S. gallolyticus subsp *gallolyticus*	5			5	0	0		0	5	0	0
S. gallolyticus subsp *pasteurianus*	6			5	1	0		0	6	0	0
S. lutetiensis	4	1	1	2	0	0		2	2	0	0
Overall	124			75	10	8		17	74	2	25

ID, identification.
[a]*sodA* sequence analysis was used as the reference method to characterize the strains.
[b]Results were considered concordant when identification by MALDI-TOF/API 20 strep exactly matched identification by the *sodA* gene sequence analysis.
[c]Discrepant results include major discrepancies (incorrect genus) and minor discrepancies (correct genus, incorrect species)

TABLE 4. Agreements for each group of VGS in identification to species level and group level[a]

	Agreement for identification to species level (%)			Agreement for identification to group level		
	API	MALDI	p[a]	API	MALDI	p[b]
mitis group (*n* = 38)	65.8 (48.6–80.4)	55.2 (38.3–71.4)	1	65.8 (48.6–80.4)	92 (78.6–98.3)	0.013
salivarius group (*n* = 12)	75 (42.8–94.5)	83.3 (51.6–97.9)	1	75 (42.8–94.5)	83.3 (51.6–97.9)	1
mutans group (*n* = 1)	100 (25–100)	100 (25–100)	1	100 (25–100)	100 (25–100)	1
anginosus group (*n* = 30)	36.6 (19.9–56.1)	83.3 (65.3–94.4)	<0.01	56.6 (37.4–74.5)	83.3 (65.3–94.4)	<0.001
sanguinis group (*n* = 19)	57.9 (33.5–79.7)	100 (82.3–100)	0.008	63 (38.4–83.7)	100 (82.3–100)	<0.001
bovis group (*n* = 24)	75 (53.3–90.2)	62.5 (40.6–81.2)	0.508	79 (57.8–92.9)	62.5 (40.6–81.2)	0.625
Overall (*n* = 124)	60.5 (51.3–69.1)	73.4 (64.7–80.9)	0.003	70 (57.9–75.2)	93.5 (87.8–97.2)	<0.001

[a]Agreements were calculated with a 95% confidence interval based on an exact binomial distribution.
[b]p <0.05 was considered statistically significant.

TABLE 5. Validity values for each group of VGS for identification to species and group level

	API 20 strep						MALDI-TOF					
	Species level ID				Group level ID				Group level ID			
Group	Sensitivity (%)	Specificity (%)	PPV (%)	NVP (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NVP (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NVP (%)
mitis	65.8 (48.6–80.4)	97.7 (91.8–99.7)	92.6 (75.7–99.1)	86.6 (78.2–92.7)	65.8 (48.6–80.4)	97.7 (91.8–99.7)	92.6 (75.7–99.1)	86.6 (78.2–92.7)	55.3 (38.3–39.4)	97.7 (91.8–99.7)	91.3 (72–98.9)	83.2 (74.4–89.9)	92.1 (78.6–98.3)	97.7 (91.8–99.7)	94.6 (81.8–99.3)	96.5 (90.3–99.3)
salivarius	75 (42.8–94.5)	96.4 (91.1–99)	69.2 (38.6–90.1)	97.3 (92.3–99.4)	75 (42.8–94.5)	96.4 (91.1–99)	69.2 (38.6–90.1)	97.3 (92.3–99.4)	83.3 (51.6–97.9)	89.3 (82–94.3)	45.4 (24.4–67.8)	98 (93.1–99.8)	83.3 (51.6–97.9)	89.3 (82–94.3)	45.4 (24.4–67.8)	98 (93.1–99.8)
mutans	100 (25–100)	100 (97–100)	100 (25–100)	100 (97–100)	100 (25–100)	100 (25–100)	100 (25–100)	100 (25–100)	100 (25–100)	100 (97–100)	100 (25–100)	100 (97–100)	100 (25–100)	100 (25–100)	100 (25–100)	100 (25–100)
anginosus	36.6 (19.9–56.1)	100 (96.2–100)	100 (80.5–100)	83.2 (75–89.6)	56.6 (37.4–74.5)	100 (96.2–100)	100 (80.5–100)	87.9 (80.1–93.4)	83.3 (65.3–94.4)	100 (96.2–100)	100 (86.3–100)	94.9 (88.6–98.3)	90 (73.5–97.9)	100 (96.2–100)	100 (87.2–100)	96.9 (91.2–99.4)
sanguinis	57.9 (33.5–79.7)	99 (94.8–100)	91.6 (61.5–99.8)	92.9 (86.4–96.9)	63.2 (38.4–83.7)	99 (94.8–100)	92.3 (64–99.8)	93.7 (87.4–97.4)	100 (82.4–100)	100 (96.6–100)	100 (82.3–100)	100 (96.6–100)	100 (82.4–100)	100 (96.5–100)	100 (82.4–100)	100 (96.5–100)
bovis	75 (53.3–90.2)	100 (96.4–100)	100 (81.5–100)	94.3 (88.1–97.9)	79.2 (57.8–92.9)	100 (96.4–100)	100 (82.4–100)	95.2 (89.2–98.4)	62.5 (40.6–81.2)	100 (96.4–100)	100 (78.2–100)	91.7 (84.9–96.2)	100 (85.7–100)	100 (96.4–100)	100 (85.7–100)	100 (96.4–100)

Validity values were calculated with a 95% confidence interval based on an exact binomial distribution.

of works, *sodA* sequence analysis has proven to be a highly accurate identification tool for characterization of VGS [3–6]. In the present study all clinical isolates of VGS recovered from blood cultures were identified to the species level by *sodA* sequence analysis. In addition, all reference strains tested were correctly identified. Therefore, we used this technique as a reference method to assess the ability of MALDI-TOF and API 20 strep to identify VGS causing blood stream infections.

Our study demonstrates that MALDI-TOF is more sensitive than API 20 Strep for identification of VGS to species level, except for species belonging to *mitis* and *bovis* groups. MALDI-TOF also shows higher sensitivity for identification of VGS to group level.

All strains that could not be discriminated at any taxonomic level by the API system were assigned to species level by MALDI-TOF. Agreement to species level between MALDI-TOF and *sodA* sequence analysis was better than that obtained between API 20 strep and *sodA* sequence analysis (73.4% vs. 60.5%, p 0.003). MALDI-TOF was also superior in identifying strains to group level (93.5% vs. 70.3%, p <0.001). API 20 strep better identified species belonging to the *mitis* and *bovis* groups, although the differences did not reach statistical significance. Identification was unreliable in only in 6/124 (4.8%) isolates (score ≤1.7) by MALDI-TOF, whereas API 20 strep was not able to identify 12/124 (9.6%). No very major discrepancies were observed with either of the two techniques. In the case of API 20 strep, 44% of discrepancies were minor (the isolate was assigned to a different species but to the same group); with MALDI-TOF, 96% were minor. We suggest that minor discrepancies can be reported to clinicians as species belonging to a specific group that is actually associated with different diseases and patterns of antibiotic resistance. MALDI-TOF was able to identify 94% of the isolates to group level while the API system was only able to identify 70% to group level. Previous reports show the poor sensitivity of API 20 strep and other phenotypic methods for identification of VGS [13,14]. Hoshino *et al.* [4] reported 50% concordance between API 20 strep and molecular identification of non-haemolytic strains of streptococci. This finding is consistent with the results shown in the present study.

Freidrichs *et al.* [10] compared MALDI-TOF and RAPID-strep/PCR16s by analysing 99 VGS isolates and 10 reference strains and demonstrated 100% consistency between MALDI-TOF and the phenotypic/genotypic identification system. However, we found that MALDI-TOF was limited in that it identified eight isolates of *S. oralis* and six isolates of *S. mitis* as *S. pneumoniae*. Misidentification of *S. mitis* group isolates as *S. pneumoniae* by MALDI-TOF has been reported elsewhere [8,9]. In our study, we ruled out all pneumococcal isolates on the basis of the optochin susceptibility test and

bile solubility test, both of which proved to be highly sensitive and specific for the identification of pneumococci when performed together [11,12]. Moreover, based on this identification scheme, in none of the cases did the reference standard (*sodA* sequence analysis) identify as *S. pneumoniae* any isolate included in the study as VGS.

Romero *et al.* [15] reported a limitation of MALDI-TOF (Bruker) in discriminating the two main *S. gallolyticus* subspecies, a finding that differs from our results. We found that five isolates of *S. gallotyticus* subsp *gallolyticus* and six isolates of *S. gallolyticus* subsp *pasteurianus* were correctly identified by MALDI-TOF. However, we also observed that nine isolates of *S. equinus* were identified as *S. gallolyticus* subsp *pasteurianus*. Therefore, we suggest that the database of Bruker technology (Biotyper 3.0 software package) should be reviewed to resolve problems associated with identification of *S. equinus* and we propose reporting these results as members of the *S. bovis* group until an appropriate solution has been found.

As for the identification scores given by the manufacturer, we assessed the level of agreement in the score interval proposed for identification to genus level (1.7–1.9). We observed that within this score interval there were 17 concordant results and 11 discrepant results, that is, 60.7% agreement in this interval score (vs. 82.2% in the ≥2 interval score). Among these 11 discrepancies, eight *S. mitis* were misidentified as *S. pneumoniae*. Although this is a limitation of this method, we suggest that VGS isolates identified by MALDI-TOF with scores between 1.7 and 1.9 could be correctly identified. However, if the identification obtained is *S. pneumoniae* additional tests should be performed.

We also propose the following identification scheme for VGS isolates from blood cultures. First, MALDI-TOF must be performed in combination with the previously mentioned tests to rule out pneumococci and enable identification of VGS to group level in 94% of isolates with 73% concordance to species level. If the score by this technique is <1.7, which in our experience occurs in 5% of cases, *sodA* sequence analysis should be performed.

In conclusion, our data show that API 20 strep does not accurately identify all isolates of VGS causing bloodstream infections. MALDI-TOF appears to be a rapid and reliable alternative for group-level identification of VGS isolated from blood cultures, although it is not able to discriminate genetically related species of certain groups and can misidentify *S. mitis* and *S. oralis* isolates as *S. pneumoniae*.

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Transparency Declaration

This study does not present any conflicts of interest for the authors.

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